APPLICATION

FOR

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TITLE: HIGH AFFINITY INTEGRIN POLYPEPTIDES AND USES
THEREOF

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HIGH AFFINITY INTEGRIN POLYPEPTIDES AND USES THEREOF

RELATED APPLICATION INFORMATION

This application claims priority from provisional application serial no. 60/221,950, filed July 31, 2000, the contents of which is hereby incorporated by reference.

BACKGROUND

Integrins are heterodimeric receptors that mediate a wide variety of important interactions both between cells and between cells and the extracellular matrix via ligand binding. All integrins have an α subunit and a β subunit. Within the α subunit a region referred to as the A domain (or I domain) is known to be an important mediator of ligand binding. A similar region, the A-like domain, is present in many β subunits. Many integrins are thought to exist in two conformations, a low affinity state (the "closed" or "unliganded" conformation") and a high affinity state (the "open" or "liganded" conformation), the later of which is responsible for high affinity ligand binding.

Integrins transduce signals that mediate the effects of the matrix on the physiological activity of cells (e.g., motility, proliferation, differentiation). Moreover, integrins play a role in inflammation and in oncogenic cell transformation, metastasis, and apoptosis. Thus, there is considerable interest in identifying compounds that can activate or inhibit the activity of one or more integrins.

In order for an efficient integrin-ligand binding to occur, it is thought that the integrin must be in its high affinity configuration. It appears that inside-out signals generated when cells are activated by a variety of stimuli apparently switch integrins from a low affinity state to a high affinity state. This functional upregulation is associated with conformational changes in the extracellular regions of integrins that include the A domain of the α subunit and the A-like domain of the β subunit (Smith et al. 1988 *J. Biol. Chem.* 263:18726).

The integrin A-domain assumes a dinocleotide-binding fold (Lee et al. 1995 *Cell* 80:631; Emsley et al. 1997 *J. Biol. Chem.* 272:28512; Li et al. 1998 *J. Cell Biol.* 143:1523; Nolte et al. 1999 *FEBS Lett.* 452:379; and Legge et al. 2000 *J. Mol. Biol.* 295:1251), with a metal ion dependent adhesion site (MIDAS) on its top, and is connected through a C-terminal

α7 helix at its bottom to the body of the integrin. MIDAS and its surrounding exposed side-chains form the binding site for physiologic ligands (Li et al., *supra*; Michishita et al., *Cell* 72:857-867, 1993; Kamata et al., *J. Biol. Chem.* 269:26006-26010, 1994; Kern et al., *J. Biol. Chem.* 269:22811-6, 1994; Edwards et al., *J Biol Chem.* 273:28937-44, 1998; Zhang et al.,

5   *Biochemistry* 38:8064-71, 1999) and certain antagonists (Rieu et al., *J Biol Chem.* 271:15858-15861, 1996). In the "open" conformation, three non-charged resides in the protein directly coordinate the metal ion in MIDAS, a pseudoligand or ligand glutamate residue (Lee et al., *supra*; Li et al., *supra*; Emsley et al., *Cell* 100:47-56, 2000) completes metal coordination. In the "closed" form, the amphipathic C-terminal α 7 helix is shifted

10   upwards by 10Å compared to the "open" form, wrapping around the rest of the domain. This large shift is associated with a change in metal coordination, where one of the three coordinating residues, a threonine, is now replaced with an aspartate, and a water molecule replaces the glutamate in completing the metal ion coordination sphere (Lee et al., *Structure* 3:1333-1340, 1995). These changes in metal coordination and topology of MIDAS are

15   similar to those described in the structurally homologous G proteins (Lee et al., *supra*).

The crystal structure of four integrin A-domains (CD11b, CD11a, CD49a and CD49b) have been reported to date (Lee et al., *supra*; Lee et al., *Structure* 3:1333, 1995; Emsley et al., *supra*; Li et al., *supra*; Emsley et al., *J Biol Chem* 272:28512, 1997). All with the exception of integrin CD11b A-domain (11bA), were found only in the "closed" form,

20   leading to the suggestion that the "open" form is a non-informative crystal artifact (Baldwin et al., *Structure* 6:923-935, 1998). Three studies support the view that the "open" form of the integrin A-domain equates with the "high" affinity state (Li et al. 1998 *J. Cell Biol* 143:1523; Rieu et al., *supra*; Oxvig et al., *Proc Nat'. Acad Sci USA* 96:2215-20, 1999). In the first, point mutations in CD11bA that are predicted on structural grounds to destabilize the

25   "closed" structure, increased the proportion of the "high affinity" form in solution (Li et al. 1998 *J. Cell Biol* 143:1523). In the second, the binding site for an "activation-dependent" monoclonal antibody mapped to a conformationally sensitive region of the A-domain (Oxvig et al., *supra*). The third study showed that an A-domain in complex with a short collagen peptide assumed the "open" conformation, and suggested that the "open" form can only be

30   obtained in the presence of ligand (Emsley et al., *supra*). While it has been suggested that

the ligand causes the conformational change in integrins, at least one study suggests that integrins can exist in high affinity state even in the absence of ligand (Smith and Cheresh 1988 *J Biol Chem* 263:18726-31). In addition, several studies suggested that ligand binding affinity in heterodimeric integrins can be altered in an allosteric manner (Li et al. 1998 *J Cell*

5   *Biol* 143:1523; Edwards et al, *J Biol Chem* 273:28937-28944, 1998; Calderwood et al, *J Biol Chem* 273:5625, 1998; Zhang et al., *J Biol Chem.* 271: 29953-7, 1996).

SUMMARY

The invention features polypeptides comprising all or part of a variant integrin α subunit A domain or a variant integrin β subunit A-like domain. In the polypeptides of the

10   invention, referred to as variant integrin polypeptides, an important isoleucine residue (described in greater detail below) is absent. The isoleucine can be either deleted or replaced with different amino acids residue, preferably a smaller or less hydrophobic amino acid residue, e.g., alanine or glycine. Because the variant integrin polypeptides of the invention tend to exist in solution in a high affinity conformation, they are useful in screening assays

15   for the identification of molecules that bind to (and/or modulate the activity of) an integrin. They are also useful for generating antibodies, e.g., monoclonal antibodies, which bind to the high affinity form of an integrin. Some such antibodies recognize an epitope that is either not present or not accessible on an integrin that is in a lower affinity conformation. Thus, the invention features antibodies which bind with greater affinity to a variant integerin of the

20   invention than to corresponding wild-type integrin. The variant integrin polypeptides of the invention can be derived from any integrin α subunit or any integrin β subunit. The variant integrin polypeptides preferably include a ligand-binding portion of an A-domain or an A-like domain.

The invention also features methods for identifying a compound that binds to a

25   variant integrin polypeptide of the invention. Such screening methods can entail exposing the polypeptide to a test compound of interest and determining whether the compound binds to the polypeptide. Thus, the assay can be a simple binding assay (e.g., where binding of the compound is measured only in the absence of the ligand) or a competitive binding assay (e.g., where binding of the compound is measured in the presence of the ligand). The

invention also features methods for identifying a compound that interferes with the binding of an integrin ligand to an integrin by measuring the binding of an integrin ligand to a variant integrin polypeptide in the presence and absence of a test compound. The ability of a test compound to interfere with the binding of an integrin ligand to an integrin may also be

5 tested. In addition, the binding specificity of a compound can be assessed by measuring the binding of the integrin ligand to a second integrin (or measuring the binding of a second ligand to the integrin) in the presence and absence of the test compound.

The invention also features methods for interfering with the binding of an integrin to an integrin ligand by administering a variant integrin polypeptide of the invention or an

10 antibody that selectively binds to a variant integrin polypeptide of the invention.

The invention also features methods of administering a variant integrin polypeptide of the invention or an antibody that selectively binds to a variant integrin polypeptide of the invention for the purpose of identifying the presence of a ligand which binds to an active integrrin. Such assays are useful for diagnosing inflammation, e.g., occult inflammation (e.g.

15 abscess or an active atherosclerotic lesion).

The invention further features nucleic acid molecules (e.g., mRNA and DNA) encoding a polypeptide of the invention or a polypeptide which includes a polypeptide of the invention. The invention also includes nucleic acid molecule encoding a fusion polypeptide comprising a polypeptide of the invention and a second polypeptide, e.g., an immunoglobulin

20 constant domain.

The experiments described below concern the design a preparation of a variant form of CD11b (an integrin α subunit) that is more active than the wild-type form of CD11b. Without being bound by any particular theory, it appears that, in solution, the amount of this variant subunit that is in the open (active) conformation is greater than for the corresponding

25 wild-type form of the subunit.

Creation of the variant CD11b involves deletion or substitution of an invariable C-terminal Ile residue. Deletion or substitution of the Ile residue confers a high affinity phenotype in isolated CD11b A domain as well as in the intact integrin. Moreover, The Ile-modified A-domain can be crystallized in the "open" conformation. Thus, without being

30 bound by any particular theory, it appears that an Ile-based allosteric switch controls affinity

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and conformation in the integrin A-domain. Accordingly, variant, high affinity forms of other integrin α subunits (and β subunits) can be created by deleting or substituting the corresponding highly conserved Ile residue.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

Figures 1A and 1B depict surface representation of the CD11bA domain crystal structure in its "closed" (Figure 1A) and "open" (Figure 1B) states, with the C-terminal α7 helix outlined as a gray ribbon, the isoleucine (I316) residue in black. An arrow points to the MIDAS face. MIDAS and SILEN (socket for isoleucine) lie on almost opposite ends of the A-domain structure.

Figures 1C and 1D depict magnified face views of SILEN residues (I135, L164, I236, Y267, lying within 4Å radius from I316) in the "closed" (Figure 1C) and "open" (Figure 1D) conformations. In the "closed" form (Figure 1C), I316 sidechain is wedged in SILEN; L312 is seen on top. L312 moves to cover SILEN in the "open" structure (Figure 1D). Figures 1A-1D were built using GRASP (Barry Honig, Columbia University, NY).

Figures 1E and 1F depict stereo views of the I316 coordination socket in the "closed" (Figure 1E) and "open" (Figure 1F) conformations. SILEN residues and I316 and L312 are labeled.

Figures 2A and 2B depict $2F_0$-F_C electron density maps of the C-terminal portions of α7 from 11bA$^{123-321}$ (Figure 2A) and 11bA$^{123-315}$ structures. The maps were contoured at 1.1σ, and made with O.

Figures 2C and 2D depict ribbon diagrams showing the complete superimposition of the 11bA$^{123-321}$ (Figure 2C, red tracing) and 11bA$^{123-315}$ (Figure 2D, red tracing) structures on the reported "closed" (Figure 2C, green tracing) and "open" (Figure 2D, green tracing) forms of the CD11b A domain, respectively. The metal ion is shown as a purple sphere.

Figures 2E and 2F depict stereo views of MIDAS in the 11bA$^{123-321}$ (Figure 2E) and 11bA$^{123-315}$ (Figure 2F) structures. The direct D242-metal bond, and indirect T209-water-

metal bond (Figure 2E) are characteristic of the "closed" conformation. The meta lion in this case is Mn^{2+}. The MIDAS conformation (a direct T209-metal bond, indirect D242-water-metal bond, and a pseudo ligand glutamate, occupying the active site, shown in green, and directly coordinating the metal ion (Ca^{2+}) are those of the "open" conformation). In Figures 2E and 2g, the metal ion is shown in purple, and hydrogen bonds as dotted yellow lines.

Figures 3A-3J depict the results of functional analysis of $11bA^{E-G}$ and $11bA^{123-315}$ A-domains using BIAcore™ for recording the interactions of $11bA^{E-G}$ (Figures 3A-3E) and $11bA^{123-315}$ (Figures 3F-3J) with the activation-dependent ligands iC3b (Figures 3A and 3F), fibrinogen (Figures 3B and 3G) or CD54 (Figures 3C and 3H). All three ligands bound to $11bA^{123-315}$, but not to $11bA^{123-321}$. The observed differences in binding were not due to differences in protein concentration, as binding to the activation-independent ligands, NIF (Figures 3D and 3I) and mAb 904 (Figures 3E and 3J) were comparable. To quantitatively determine the affinity, various concentrations of the A domain peptides were used. The binding data were analyzed by linear transformation, giving dissociation constants (Kd, mean±SD, n=2) of 0.46±0.15 µM (for iC3b), 0.25±0.07 µM (for fibrinogen); and 0.22±0.04 µM (for CD54).

Figure 4A depicts the results of an analysis of the binding of $11bA^{I \to G}$ to iC3b. The dotted line represents the lack of binding of $11bA^{123-321}$ to the same ligand. The calculated Kd is 0.66±0.3 µM (mean ± SD, n=2).

Figure 4B depicts a histogram (mean ± SD, n=3) showing the relative binding of $^{I \to G}$ CR3 expressed on COS cells to iC3b, compared with wild-type receptor.

Figure 5 depicts an alignment of the A domains of nine alpha integrin α subunit (CD11b, CD11c, CD11d, CD11a, alpha 1, alpha 2, alpha 10, alpha 11, and alpha E). In this alignment, the invariant Ile (I316) is indicated by an arrow.

Figure 6 shows that an I to G mutation of the invariant isoleucine in CD11a A domain induces an activated domain in this integrin, underscoring the applicability of the findings in the CD11b A domain to other integrin A-domains.

Figure 7 is an alignment of the A-like domains of eight integrin β subunits. In this alignment, the residue corresponding to the invariant Ile in β subunits is indicated by an arrow.

DETAILED DESCRIPTION

Example 1: Generation of a stable, high affinity CD11b variant A-domain by deletion

A variant CD11b A domain consisting of amino acids E^{123} to K^{315} of CD11b ($11bA^{123-315}$; also referred to as $11bA^{E-K}$) was created. Note that the amino acid numbering in these example corresponds to the mature protein. While the amino acid numbering in Tables 2 nad 3 belwo referes to the numbering in the complete protein (including signal sequence). CD11b has a 16 amino acid signal sequence. Thus, E^{123} in the mature protein corresponds to E139 in the complete protein. This variant A domain was characterized and compared to a CD11b A domain consisting of amino acids E^{123} to G^{321} ($11bA^{123-321}$; also referred to as $11bA^{E-G}$). A variant A domain with a Ile to Gly change at residue 316 ($11bA^{I \rightarrow G}$) was also created. All three proteins were expressed as GST fusion proteins that were cleaved to release the protein of interest.

The variant polypeptides were created using standard recombinant techniques. Restriction and modification enzymes were purchased from New England Biolabs, Inc. (Beverly, MA), Boehringer Mannheim (Germany), or GIBCO BRL (Gaithersburd, MD). Site-directed mutagenesis was carried out in pGEX-4T-1 vector as described (Rieu et al. 1996 *J Biol Chem* 271:15858). The following mutagenic primers were used. IFAdel Fwd: 5'-TATAGGATCCGAGGCCCTCCGAGGGAGTCCTCAAGAGGATAG-3'; Reverse: 5'-CTACTCGAGTTACTTCTCCCGAAGCTGGTTCTGAATGGTC-3'; I-G reverse: 5'-CTACTCGAGTTAACCCTCGATCGCAAAGCCCTTCTC-3'. Introduction of the respective mutation was confirmed by direct DNA sequencing. The PvuI-BspEI-restricted cDNA fragment of the A-domain containing the mutation was subcloned into the PvuI-BspEI-restricted CD11b cDNA, cloned into pcDNA3 plasmid, which containing full-length human CD11b (Rieu et al. 1996 *J Biol Chem* 271:15858). 11b $A^{123-321}$ and $11bA^{123-315}$ and $11bA^{I \rightarrow G}$ A-domains were expressed as GST fusion proteins in *Escherichia coli* (Michishita et al. 1993 Cell 72:857), cleaved with thrombin and purified as described Li et al. 1999 *J. Cell Biol* 143:1523. C^{129} was replaced by S in all the expressed GST-A-domain fusion form to prevent formation of disulfide-linked dimmers in solution after thrombin cleavage (not shown). Purity was confirmed by SDS-PAGE analysis.

The structures of 11bA[123-321] and 11bA[123-315] were determined by x-ray crystallography. Crystals were grown using 10mg/ml stock protein solutions and the hanging drop vapor diffusion method as described Li et al. (1998 *J. Cell Biol* 143:1523). Several crystal conditions were tried for each A-domain. 11bA[123-315] and 11bA[1→G] formed crystals in the presence of a reservoir solution containing 15% polyethylene glycol 8 K, 0.10 M Tris, pH 8.2, 150 mM $CaCl_2$, at room temperature. Crystals started to form within a week, grew to a typical size of 0.3 mm x 0.05 mm x 0.04 mm in two weeks, and belonged to the tetragonal space group P4, with unit cell of a=b=45.7Å. 11bA[123-321] did not crystallize under these conditions, but formed crystals at room temperature using 10% polyethylene glycol 4000, 0.1M sodium citrate, pH 4.5, 5 mM $MnCl_2$ in the reservoir buffer. 11bA[123-321] was crystallized in the $P2_12_12_1$ space group with unit cell of a =48.1 Å, b = 121.5 Å, c = 74.5 Å.

A single 11bA[123-315] crystal was used to collect a 2.3 Å resolution data set, at 100 K, on beamline X12B of the National Synchrotron Light Source at the Brookhaven National Laboratory using a CCD detector. A single 11bA[123-321] crystal was used to collect a 2.6Å resolution data set, at 100K, using in-house X-ray on image plate. DATA were processed with DENZO and SCALEPACK to an Rsym of 8.8%, 7.7%, respectively. The starting models were the refined 1.8 Å Mg^{2+} structure (pdb accession code 1ido) (Lee 1995 *Cell* 80:631) comprising residues D132 to K315, and the refined 2.0 Å Mg^{2+}-structure (pdb accession code 1jlm) (Lee et al. 1998 *Structure* 6:923), comprising residues D132 to A318, with the metal and water molecules removed, respectively. The preliminary rigid body refinements were performed using X-plor, with the diffraction data range from 8.0 to 3.0Å resolution with 5% of reflections for R-free calculation. The phases were gradually extended to high resolution and the structures were refined by several cycles of alternating torsion-angle dynamics and restrained individual isotropic B factor refinement protocols with all diffraction data between 8.0 and 2.6 Å, and 8.0 and 2.3Å resolution, for 11bA[123-321] and 11bA[123-315] structures, respectively (Table 1). Model inspection and manual adjustments were made on SGI graphics workstations using O (Jones et al. 1991 *Acta Crystallogr* 47:110). The addition of solvent molecules was based on the suitable peaks in difference maps, reasonable hydrogen bond and refined temperature factors of less than 50 $Å^2$. The addition of solvent molecules was based on the suitable peaks in difference maps, reasonable

hydrogen bond and refined temperature factors of less than 50 Å^2. The structure was refined to a final R factor of 20.3% (R-free: 25.6%) for the 11bA$^{123-315}$ structure, and 21.9% (R-free: 30.0%) for the 11bA$^{123-321}$ structure. The final models comprises all nonhydrogen atoms of residues D132 to K315, 30 water molecules, and one Ca^{2+} ion for 11bA$^{123-315}$, and residues D132 to G321, 44 water molecules and one Mn^{2+} ions for 11bA$^{123-321}$. Crystallographic data is presented in Table 1.

Table 1

	11bA$^{123-321}$	11bA$^{123-315}$	11bA$^{I316 \to G}$
Space group	P2$_1$2$_1$2	P4$_3$	P4$_3$
Unit cell constants (Å)	a=48.1, b=121.5, c=74.6	a=b=45.7 c=94.8	a=b=45.2, c=95.0
No. of unique reflections	13,990	7,955	3,749
Resolution (Å)	8.0 - 2.6	8.0 - 2.3	8.0 – 3.0
R$_{merge}$ (%)*	7.7 {23}§	8.8 {28} §	11.2 {30.4}§
Completeness (%)	99.3 {98.4}§	91.1{80.8}§	95.3 {73.6}§
Redundancy	5.9	2.3	2.7
R-factor ^{I} (%)	21.9	18.8	20.8
R-free ‡ (%)	30.0	24.8	28.8
Solvent molecules	82	62	20
Metal ions	2 (Mn)	1 (Ca)	1 (Ca)
Root mean square (rms) deviations from ideal values			
Bond lengths (Å)	0.007	0.007	0.006
Bond angle (°)	1.3	1.3	1.25

*R$_{merge}$ =Σ|I-⟨I⟩|/Σ I where I is the observed intensity and <I> is the average intensity from multiple observations of symmetry-related reflections.

§ Numbers in {} shows values for highest 0.1 Å resolution bin.
^{I} R-factor= Σ| Fo-Fc|Σ Fo.
‡ R-free = Σ$_T$ | Fo-Fc|Σ$_T$ Fo, where T is a test set containing a randomly selected 5% of the reflections omitted from the refinement.

The crystal structure 11bA$^{123-321}$ was that of the "closed" conformation (Figures 2A, 2C, and 2E). In contrast, 11bA$^{123-315}$ crystallized in the "open" form (Figures 2B, 2D, and 2F).

The ligand binding properties of 11bA$^{123-321}$ and 11bA$^{123-315}$ were determined using surface plasmon resonance as previously described (Li et al., *supra*) on a BIAcore (BIAcore AB, Uppsala, Sweden). IC3b, fibrinogen or CD54 each was covalently couples via primary

amine groups to the dextran matrix of a separate CM5 sensor chip (BIAcore AB). BSA immobilized in the same way was used as a control surface. $11bA^{123-321}$, $11bA^{123-315}$ and $11bA^{I \rightarrow G}$ A-domains were flowed over the chip at 5 ml/min at different times. TBS (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 2 mM $MgCl_2$ and 0.005% P20 (BIAcore AB) was

5   used as the running buffer. 1 M NaCl in 20 mM Tris-HCl, pH 8.0, was used to remove the bound proteins and to regenerate the surface. Binding was measured as a function of time. The binding data (after subtracting background binding to BSA-coated chip) were analyzed using Scatchard plots as described (Dall'Acqua et al. 1996 *Biochemstry* 35:9667-9676). COS M7 simian fibroblastoid cells at ~70% confluence were transfected with supercoiled cDNAs

10   encoding WT or $CD11b^{I \rightarrow G}$ together with full-length CD18 as described (Michishita et al., *supra*)

        Transfected COS cells were grown for 24 h in Iscove's modified Dulbecco's medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% FBS, 2 mM glutamine, 50 IU/ml penicillin and streptomycin at 37°C. Cells were washed, detached with 0.1% trypsin-

15   EDTA, and seeded in replicates for 24 h onto 24- or 48-well plates (Costar Corp., Cambridge, MA) or 100-mm petri dishes. Confluent monolayers in 24- or 48-well plates were used for cell-surface antigen quantification and ligand-binding studies, and those on petri dishes for immunoprecipitation studies. Heterodimer formation and binding of iC3b-coated erythrocytes to wild type and $CR3^{I \rightarrow G}$ holoreceptors were carried out as described

20   (Rieu et al. 1996 *J Biol Chem* 271:15858). Specific binding of iC3b to the holoreceptors was obtained by subtracting background binding to mock-transfected COS cells. Binding to $CR3^{I \rightarrow G}$ was expressed as a percentage of binding to wild type, after correcting for the degree of surface expression using binding of mAb904 (Rieu et al. 1996 *J Biol Chem* 271:15858).

        As shown in Figures 3A-3J, $11bA^{123-321}$ showed no binding to the activation-

25   dependent "physiologic" ligands, complement iC3b, firbrinogen and CD54 (ICAM-1). In contrast, $11bA^{123-315}$ displayed high affinity binding to all three ligands (Figures 3A-3J). Both domains bound equally well to the activation-independent "ligands" NIF (neutrophil inhibitory factor) (Figures 3D, and 3I), and mAb 904 (Figures 3E and 3J), indicating that the differences observed are not caused by variations in A-domain concentrations. These data

conclusively establish that the "open" and "closed" crystal structures correspond respectively to the "high" and "low" affinity states of integrin 11bA.

Example 2: Generation of a stable, high affinity state CD11b variant by substitution

Ile^{316} is invariable in all integrin alpha A-domains cloned to date (Figure 5). An A-
5   domain with an Ile to glycine substitution (11bA$^{I \to G}$) exhibited a "high affinity" state (Figure 4A). The same substitution created in the holoreceptor dramatically increased its ligand binding activity (Figure 4B). The crystal structure 11bA$^{I \to G}$ was identical to that of the "open" 11bA$^{123-315}$ form. These data firmly establish that the "open" high affinity conformation is primarily dictated by an Ile-based switch, intrinsic to the domain, and acting
10   allosterically to regulate ligand binding affinity on the MIDAS face. It is known that the inactive an active conformers of an integrin exist in the absence of ligand (Smith et al., *supra*; Yan et al., *J. Biol. Chem.* 275:7249-60, 2000). Based on the results described above, it appears that the role of the ligand is not in initiating the high affinity state as has been recently proposed (Emsely et al, supra), but in stabilizing it, through shifting the equilibrium
15   between the low and high affinity state in favor of the latter (Li et al., *supra*). Integrin activation by inside- out signaling would lead to a shift in this equilibrium, increasing the proportion of high affinity receptors on the cell surface that become "available" for ligand binding. Ligand engagement would then generate new epitopes, perhaps extrinsic to the A-domain, that initiate outside-in signaling.
20   A conserved hydrophobic intramolecular socket (SILEN, Socket for Isoleucine), fastens the I^{316} finger in the "closed" conformation; I^{316} is replaced by L^{312} in the "open" structure (Figures 1A and 1F). SILEN is formed by the hydrophobic side chains of I^{135}, L^{164}, I^{236}, and Y^{267} both in the "closed" and "open" conformations. (Figures 1C and 1D). In several integrins, certain mutations that lie outside MIDAS produce gain-of function effects
25   in the holoreceptor, and are believed to act allosterically (Zhang et al., *supra*; Oxvig et al., *supra*; Zhang et al., *supra*). These studies were carried out in the holoreceptors, making it difficult to provide a mechanistic interpretation, because of potential interdomain interactions and/or other quaternary effects. These mutations occur in or around SILEN. For example, substitution of the α1-βB loop of CD11b with that of CD11a, generates a constitutively
30   active integrin (Zhang et al., *supra*). This region spans one of the SILEN residues L^{164}.

Integrin activation also occurs in an L^{164}-F substitution (Oxvig et al, supra), which predictably makes SILEN smaller and therefore less accommodation to I^{316}. Other activating mutations involving E^{131}, D^{132}, K^{231} and F^{234} lie at the bottom of the structure, in close proximity to SILEN (Oxvig et al., *supra*), and may thus exert their effect through

5   interference with the proper coordination of the Ile "finger" in SILEN. The inhibitory effect of certain mAbs with epitopes on the opposite side of MIDAS (e.g., mAb 44a, the epitope of which spans residues on the top of SILEN) may similarly be explained through stabilization of the SILEN pocket.

The presence of the N-terminal extension facilitates A-domain switching into the less

10   favored high-affinity state (Li et al, *supra*). The underlying structural basis for this effect is unknown, since none of the residues in the N-terminal extension are included in the derived 3-D structures. It has been observed however that residues within this extension regulate ligand binding in A-domains. First, naturally occurring point mutations in this segment of the vWF A1 domain cause gain-of-function phenotypes in patients with type IIB vWf disease

15   (Matsushita et al., *J. Biol. Chem* 279:13406-14, 1995). This region also contains an activating mutation in CD11b A domain (Oxvig et al., *supra)*. Third, synthetic peptide from the N-terminal extension inhibited CD11a-dependent adhesion. Structural data from the CD49b A-domain also show that three residues that extend beyond the $\alpha 7$ helix can pack into a crevice formed in part by residues in the N-terminal extension, bringing the N and C

20   termini into very close proximity (Emsley et al., *supra*). Flexibility of the C-terminal residues in $\alpha 7$ has also been observed in the crystal and NMR structures of the CD11a A-domain. Taken together, these data suggest the N-terminal extension may offer an alternative by imperfect "competitive" surface for luring Ile away from SILEN, allowing some molecules to exist in the "open" form. The present data suggest that such a mechanism

25   may operate in the holoreceptor, providing a mechanistic basis for integrin activation by inside-out signals.

Variant Integrin Polypeptides

Given the sequence similarity among the A domains of integrin α subunits, deletion of substitution of the Ile in a selected integrin α subunit that corresponds to Ile^{316} of CD11b

should result in the creation of a variant integrin α subunit that is more active (i.e., in solution has a greater proportion of ligand binding form polypeptides) that the wild-type form of the subunit. Figure 5 is an alignment of the C-terminal α7 helix of the A domains of nine integrin α subunits (CD11b, CD11c, CD11d, CD11a, alpha 1, alpha 2, alpha 10, alpha

5   11, and alpha E). In this alignment, the invariant Ile corresponding to Ile[316] of CD11b in the other integrin α subunits is outlined (arrow). Replacing the invariant Ile with Ala or Gly or some other suitable amino acid should create a variant integrin polypeptide with increased activity. We have shown this to be the case in the CD11a A-domain (Figure 6). A variant CD11a A domain containing an I to G substitution displays binding to the activation

10   dependent ligand ICAM-1 in an ELISA assay. No binding was observed in the wild-type protein without this substitution. Alternatively, the portion of the integrin α subunit (or the A domain) that includes the invariant Ile and all amino acid residues C terminal to the invariant Ile can be deleted. Table 2 below lists the position of the invariant Ile in each of the integrin α subunits depicted in Figure 5.

15   **Table 2**

Integrin α Subunit	GenBank Accession No.	Invariant Ile Position in whole integrin	A domain
Human CD11b	RWHU1B	Residue 332	C144-A334
Human CD11c	RWHU1C	Residue 333	C145-A335
Human CD11d	AAB38547	Residue 332	C144-A334
Human CD11a	AAC31672	Residue 331	C150-V333
Human Alpha 1 (CD49a)	P56199	Residue 331	C139-A333
Human Alpha 2 (CD49b)	NP_002194	Residue 361	C169-S363
Human Alpha 10	XP_002097	Residue 249	C57-G251
Human Alpha 11	NP_036343	Residue 349	C159-S351
Human Alpha E	A53213	Residue 385	E196-S387

The present invention features variant integrin α subunit polypeptides in which the invariant Ile (listed in Table 2) is substituted by Gly, Ala or some other amino acid (e.g., Val). The polypeptide can include part or all of the indicated A domain, e.g., 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 contiguous amino acids of

5 the A domain that includes the position of the invariant Ile. The invention also includes variant integrin α subunits in which the Ile has been deleted. Also within the invention are polypeptides comprising the entire A domain of an integrin α except for the invariant Ile. For example, amino acids 144-132 of CD11b, amino acids 145-332 of CD11c, amino acids 144-331 of CD11d, amino acids 150-330 of CD11a, but does not include the remainder of the

10 integrin α subunit. The invention also features polypeptides comprising the A domain of an integrin α subunit up to but not including the invariant Ile and further lacking the 5 amino acids following the invariant Ile (e.g., amino acids 144-331, but not 332-336 of CD11b; amino acids 145-332, but not 333-337 of CD11c; amino acids 144-331, but not 332-336 of CD11d; amino acids 150-330, but not 331-335 of CD11a; amino acids 139-330, but not 331-

15 335 of human alpha 1; amino acids 169-360, but not 361-335 of human alpha 2; amino acids 57-248, but not 249-253 of human alpha 10; amino acids 159-348, but not 349-353 of human alpha 11; or amino acids 196-384, but not 385-389 of human alpha E).

Given the sequence similarity among the A-like domains of integrin β subunits, deletion or substitution of the Ile in a selected integrin β subunit that corresponds to Ile[316] of

20 CD11b should result in the creation of a variant integrin β subunit that is more active (i.e., in solution has a greater proportion of ligand binding form polypeptides) than the wild-type form of the subunit. Figure 7 is an alignment of the A-like domains of integrin β subunits. Replacing the conserved Ile with Ala or Gly or some other suitable amino acid should create a variant integrin polypeptide with increased activity. Alternatively, the portion of the

25 integrin β subunit (or the A-like domain of the subunit) that includes the conserved Ile and all amino acid residues C terminal to the invariant Ile can be deleted. Table 3 below lists the position of the conserved Ile in each of the integrin β subunits depicted in Figure 7.

Table 3

Integrin β Subunit	GenBank Accession No.	Conserved Ile Position	A-like domain
Human β2(CD18)	XP_009806	L363	Y125-S365
Human β1	NP_002202	L378	Y141-S380
Human β3	I77349	I377	Y136-S379
Human β4	AAC51632	I366	S128-S368
Human β5	A38308	I378	Y136-S380
Human β6	NP_000879	L371	Y131-S373
Human β7	NP_000880	L389	Y150-S391
Human β8	NP_002205	L384	Y145-S386

Nucleic Acid Molecules Encoding Variant Integrin Polypeptides

The invention features isolated or purified nucleic acid molecules that encodes a
variant integrin polypeptide, e.g., a full length variant integrin subunit or a fragment thereof
(e.g., in which the invariant Ile is deleted or substituted), e.g., a biologically active variant
integrin polypeptide.

In one embodiment, an isolated nucleic acid molecule of the invention includes the
nucleotide sequence encoding amino acids 123-315 of the CD11b α subunit (SEQ ID NO:1)
or a portion thereof. The nucleic acid molecules can include non-coding sequences or
sequences encoding all or a portion of a protein other than an integrin.

In another embodiment, an isolated nucleic acid molecule of the invention includes a
nucleic acid molecule which is a complement of the nucleotide sequence encoding amino
acids 123-315 of the CD11b α subunit.

A nucleic acid molecule of the invention can include only a portion of the nucleic acid
sequence of SEQ ID NO:1. For example, such a nucleic acid molecule can include a
fragment which encodes all or a portion (e.g., an immunogenic or biologically active portion)
or an integrin A domain or A-like domain.

Variant Integrin Polypeptides

The present invention also includes variant integrin polypeptides. Such polypeptides can be produced using recombinant DNA methods, by chemical synthesis or using other techniques. The polypeptides can be used as immunogens or antigens generate antibodies which bind the active form of an integrin A-domain or A-like domain. The polypeptide can be post-translationally modified, e.g., glycosylated.

A variant integrin polypeptide can be part of a fusion protein which includes all or a portion of a second polypeptide that is not a variant integrin polypeptide. This second polypeptide can be fused to the C-terminus or the N-terminus of the variant integrin polypeptide. All or part of a third peptide may also be present. Thus, a variant integrin polypeptide can be fused to, e.g., GST, an immunoglobulin constant region, a heterologous signal sequence.

The variant integrin polypeptide fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. For example, the variant integrin polypeptides can be used to reduce skeletal muscle injury. Isolated CD11b A domain has already been found to reduce damage in an animal model of this type of injury. Briefly, purified recombinant rat CD11b A-domain was administered intravenously in a single dose (1mg/kg) to seven groups of Lewis rats (5 per group), 30 minutes before inducing mechanically skeletal muscle injury. Equal numbers of rats were treated with a function-blocking anti-CD11b/CD18 mAb (1 mg/kg). Quantitative histological examination of the wounded area in controlled rats (treated with PBS), showed edema, myofiber disruption, necrosis and erythrocytes extravasation. Influx of neutrophils was detected 30 minutes post wound, followed by a second wave 3 hours later. There was also significant tissue necrosis outside the immediate wounded area (5 mm zone) associated with the presence of activated neutrophils. A-domain or mAb-treated rats showed a comparable and significant decrease in the number of infiltrated PMN (75±10%, n=35) and a protection of the muscular fibers outside the immediate zone of necrosis (80±8%, n=35). These data show that the A-domain can be an effective tissue-preserving agent in this model of muscular injury. Variant A domain should be equally effective.

The variant integrin polypeptide fusion proteins can be used to affect the bioavailability of a variant integrin polypeptide ligand.

Variant integrin polypeptide fusion proteins may be useful therapeutically for the treatment of disorders caused by, for example, ischemia-reperfusion injury (*Stroke* 30:134-9,1999), immune complexes (*J Exp Med* 186:1853-63,1997), restenosis, and parasitic diseases (e.g. *Ancylostoma* spp; see *J Cell Biol* 127:2081-91, 1994).

Moreover, the variant integrin polypeptide-fusion proteins of the invention can be used as immunogens to produce anti-variant integrin polypeptide antibodies in a subject, to purify variant integrin polypeptide ligands and in screening assays to identify molecules that inhibit the interaction of variant integrin polypeptide with an variant integrin polypeptide ligand.

Expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). An variant integrin polypeptide-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the variant integrin polypeptide protein.

Variant integrin polypeptides can be produced using an expression vectors (e.g., a plasmid vector or a viral vector). The vector can be autonomously replicating or integrated in to the host genomes. The expression vector can include at least one regulatory sequence (e.g., promoter, enhancer, polyA site, or a cell- or tissue-specific transcription factor binding site) that is operatively linked to the nucleic acid sequence to be expressed. The expression vector can be designed for expression in prokaryotic or eukaryotic cells, e.g., plant cell, insect cells, yeast cells, mammalian cells, and *E coli*. Depending on the host cells used to express the variant integrin polypeptide, it may be desirable to encode the polypeptide using codons optimized for the host cell. In some cases it may be desirable to employ an expression vector capable of directing tissue-specific expression.

The invention also features host cells or recombinant cells harboring a nucleic acid molecule encoding a variant integrin polypeptide. The nucleic acid molecule can be integrated into the host cells genome or present in an autonomously replicating vector. A host cell can be any prokaryotic or eukaryotic cell, e.g., *E. coli*, an insect cell, yeast or a

mammalian cell. The nucleic acid molecules can be introduced into the host cells through transformation or transfection techniques.

A host cell of the invention can be used to produce (i.e., express) a variant integrin polypeptide by culturing the host cell under conditions such that the polypeptide is produced and then isolating the polypeptide from the cells or the culture medium.

Antibodies Recognizing Variant Integrin Polypeptides

The invention also features antibodies directed against a variant integrin polypeptide. Such antibodies bind to the variant polypeptide with greater affinity than they bind to the corresponding wild-type integrin polypeptide. The antibodies can be generated using standard methods and can be screened by comparing the binding of the antibody to the variant integrin polypeptide to binding of the antibody to the corresponding wild-type polypeptide.

The term "antibody" refers to an immunoglobulin molecule or immunologically active portion thereof, i.e., an antigen-binding portion. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')$_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The antibody can be a polyclonal, monoclonal, recombinant, e.g., a chimeric or humanized, fully human, non-human, e.g., murine, or single chain antibody. In a preferred embodiment it has effector function. The antibody can be coupled to a toxin or imaging agent for use in diagnosis of occult inflammation (e.g. in abscess or active atherosclerotic plaques).

Chimeric, humanized, but most preferably, completely human antibodies are desirable for applications which include repeated administration, e.g., therapeutic treatment (and some diagnostic applications) of human patients.

The anti-variant integrin polypeptide antibody can be a single chain antibody which can be optionally dimerized or multimerized to generate multivalent antibodies. The antibody can be designed to have little or no ability to bind to an Fc receptor.

The antibodies of the invention can be used to detect or purify integrin subunits that are in the active conformation. Thus, they can be used to evaluate the abundance and pattern of expression of an active form of an integrin as part of a clinical testing procedure. Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable

substance (i.e., antibody labeling). Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials (e.g., horseradish peroxidase, alkaline phosphatase, β-galactosidase, acetylcholinesterase, streptavidin/biotin and avidin/biotin, umbelliferone,

5   fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin, luminol, luciferase, luciferin, aequorin, ^{125}I, ^{131}I, ^{35}S and ^{3}H).

Screening Assays to Identify Compounds that Interact with a Variant Integrin Polypeptide

The invention features methods for evaluating a compound for the ability to bind to a

10   variant integrin polypeptide or inhibit the ability of an integrin ligand (e.g., a naturally-occurring integrin ligand) to bind to an variant integrin polypeptide. The methods can include contacting the compound with the variant integrin polypeptide in the presence or absence of a ligand and measuring the ability of the compound or the ligand to bind to the variant polypeptide. The methods can be performed *in vitro*, e.g., in a cell free system, or *in*

15   *vivo*, e.g., in a two-hybrid interaction trap assay. The method can be used to identify naturally-occurring molecules which interact with an integrin. It can also be used to find natural or synthetic inhibitors of the interaction between an integrin and an integrin ligand.

The compounds tested can be, e.g., proteins, peptides, peptidomimetics, peptoids, and small molecules. The test compounds can be obtained from combinatorial libraries

20   including: biological libraries; peptoid libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; "one-bead one-compound" libraries.

Libraries of compounds may be presented in solution (e.g., Houghten 1992 *Biotechniques* 13:412-421), or on beads (Lam 1991 *Nature* 354:82-84), chips (Fodor 1993

25   *Nature* 364:555-556), bacteria (Ladner U.S. Pat. 5,223,409), spores (Ladner U.S. Pat. 5,223,409), plasmids (Cull et al. 1992 *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith 1990 *Science* 249:386-390); Devlin 1990 *Science* 249:404-406; Cwirla et al. 1990 *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici 1991 *J. Mol. Biol.* 222:301-310; Ladner, *supra.*).

The screening assay can be a cell-based assay in which a cell which expresses an variant integrin polypeptide protein or biologically active portion thereof is contacted with a test compound, and the ability of the test compound to modulate variant integrin polypeptide activity is determined. Determining the ability of the test compound to modulate variant

5   integrin polypeptide activity can be accomplished by monitoring, for example, the ability to bind an integrin ligand.

The ability of the test compound to modulate variant integrin polypeptide binding to a compound, e.g., an integrin ligand or to bind to variant integrin polypeptide can also be evaluated. This can be accomplished, for example, by coupling the compound, e.g., the

10   substrate, with a radioisotope or enzymatic label such that binding of the compound, e.g., the substrate, to variant integrin polypeptide can be determined by detecting the labeled compound, e.g., substrate, in a complex. Alternatively, a variant integrin polypeptide or the integrin ligand can be coupled with a radioisotope or enzymatic label to monitor the ability of a test compound to modulate variant integrin polypeptide binding to an integrin ligand. For

15   example, compounds can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

20   In yet another embodiment, a cell-free assay is provided in which an variant integrin polypeptide or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the variant integrin polypeptide or biologically active portion thereof is evaluated. Preferred biologically active portions of the variant integrin polypeptide to be used in assays of the present invention include fragments that bind an

25   integrin ligand.

Soluble and/or membrane-bound forms of isolated proteins (e.g., variant integrin polypeptide proteins or biologically active portions thereof) can be used in the cell-free assays of the invention. When membrane-bound forms of the protein are used, it may be desirable to utilize a solubilizing agent. Examples of such solubilizing agents include non-

30   ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-

N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

5   Cell-free assays involve preparing a reaction mixture of the target gene protein and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex that can be removed and/or detected.

The interaction between two molecules can also be detected, e.g., using fluorescence energy transfer (FET) (see, for example, Lakowicz et al., U.S. Patent No. 5,631,169;

10   Stavrianopoulos et al., U.S. Patent No. 4,868,103). A fluorophore label on the first, 'donor' molecule is selected such that its emitted fluorescent energy will be absorbed by a fluorescent label on a second, 'acceptor' molecule, which in turn is able to fluoresce due to the absorbed energy. Alternately, the 'donor' protein molecule may simply utilize the natural fluorescent energy of tryptophan residues. Labels are chosen that emit different wavelengths of light,

15   such that the 'acceptor' molecule label may be differentiated from that of the 'donor'. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, the spatial relationship between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. An FET binding event can be conveniently

20   measured through standard fluorometric detection means well known in the art (e.g., using a fluorimeter).

In another embodiment, determining the ability of the variant integrin polypeptide protein to bind to a target molecule, e.g., an integrin ligand, can be accomplished using real-time Biomolecular Interaction Analysis (BIA) as described above (see, e.g., Sjolander and

25   Urbaniczky (1991) *Anal. Chem.* 63:2338-2345 and Szabo et al. (1995) *Curr. Opin. Struct. Biol.* 5:699-705). "Surface plasmon resonance" or "BIA" detects biospecific interactions in real time, without labeling any of the interactants (e.g., BIAcore). Changes in the mass at the binding surface (indicative of a binding event) result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)),

resulting in a detectable signal which can be used as an indication of real-time reactions between biological molecules.

In one embodiment, the polypeptide or the test compound is anchored onto a solid phase. The polypeptide/test compound complexes anchored on the solid phase can be

5   detected at the end of the reaction. Preferably, the polypeptide can be anchored onto a solid surface, and the test compound, (which is not anchored), can be labeled, either directly or indirectly, with a detectable labels.

It may be desirable to immobilize either a variant integrin polypeptide, an anti-variant integrin polypeptide antibody or its target molecule to facilitate separation of complexed

10   from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to a variant integrin polypeptide protein, or interaction of an variant integrin polypeptide protein with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes,

15   and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/variant integrin polypeptide fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then

20   combined with the test compound or the test compound and either the non-adsorbed target protein or variant integrin polypeptide protein, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly

25   or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of variant integrin polypeptide binding or activity determined using standard techniques.

Other techniques for immobilizing either a variant integrin polypeptide or a target molecule on matrices include using conjugation of biotin and streptavidin. Biotinylated

30   variant integrin polypeptide or target molecules can be prepared from biotin-NHS (N-

hydroxy-succinimide) using techniques known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical).

In order to conduct the assay, the non-immobilized component is added to the coated
5   surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled, the detection of label immobilized on the surface
10   indicates that complexes were formed. Where the previously non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the immobilized component (the antibody, in turn, can be directly labeled or indirectly labeled with, e.g., a labeled anti-Ig antibody).

In one embodiment, this assay is performed utilizing antibodies reactive with variant
15   integrin polypeptide or target molecules but which do not interfere with binding of the variant integrin polypeptide to its target molecule. Such antibodies can be derivatized to the wells of the plate, and unbound target or variant integrin polypeptide protein trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of
20   complexes using antibodies reactive with the variant integrin polypeptide or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the variant integrin polypeptide or target molecule.

Alternatively, cell free assays can be conducted in a liquid phase. In such an assay, the reaction products are separated from unreacted components, by any of a number of
25   standard techniques, including chromatography, electrophoresis and immunoprecipitation.

In a preferred embodiment, the assay includes contacting the variant integrin polypeptide or biologically active portion thereof with a known compound which binds variant integrin polypeptide to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with an variant
30   integrin polypeptide protein, wherein determining the ability of the test compound to interact

with an variant integrin polypeptide includes determining the ability of the test compound to preferentially bind to variant integrin polypeptide or biologically active portion thereof, or to modulate the activity of a target molecule, as compared to the known compound.

To identify compounds that interfere with the interaction between a variant integrin
5   polypeptide and an integrin ligand, a reaction mixture containing the variant integrin polypeptide and the ligand is incubated, under conditions and for a time sufficient, to allow the two products to form complex. In order to test an inhibitory agent, the reaction mixture is provided in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture, or can be added at a time subsequent to the addition
10   of the target gene and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the variant integrin polypeptide and integrin ligand is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction. Additionally,
15   complex formation within reaction mixtures containing the test compound and variant integrin polypeptide can also be compared to complex formation within reaction mixtures containing the test compound and the corresponding wild-type integrin polypeptide.

These assays can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the variant integrin polypeptide or the
20   binding partner (i.e., the integrin ligand) onto a solid phase, and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the variant integrin
25   polypeptides and the binding partner (e.g., an integrin ligand), e.g., , by competition, can be identified by conducting the reaction in the presence of the test substance. Alternatively, test compounds that disrupt preformed complexes, e.g., compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats
30   are briefly described below.

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In a heterogeneous assay system, either the variant integrin polypeptide or the integrin ligand, is anchored onto a solid surface (e.g., a microtiter plate), while the non-anchored species is labeled, either directly or indirectly. The anchored species can be immobilized by non-covalent or covalent attachments. Alternatively, an immobilized antibody specific for the species to be anchored can be used to anchor the species to the solid surface.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g., by washing) and any complexes formed will remain immobilized on the solid surface. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, can be directly labeled or indirectly labeled with, e.g., a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds that inhibit complex formation or that disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds that inhibit complex or that disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. For example, a preformed complex of the variant integrin polypeptide and the integrin ligand is prepared in that either the variant integrin polypeptide or the integrin ligand is labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Patent No. 4,109,496 that utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result

in the generation of a signal above background. In this way, test substances that disrupt variant integrin polypeptide-binding partner interaction can be identified.

In yet another aspect, the variant integrin polypeptide proteins can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Patent No. 5,283,317;

5   Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J. Biol. Chem.* 268:12046-12054; Bartel et al. (1993) *Biotechniques* 14:920-924; Iwabuchi et al. (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with variant integrin polypeptide.

A two-hybrid assay can be carried out using a variant A domain as the bait protein.

10   Briefly, the variant A domain is fused to the LexA DNA binding domain and used as bait. The prey is an aptamer library cloned into the active site loop of TrxA expression as a fusion protein with an N-terminal nuclear localization signal, a LexA activation domain, and an epitope tag (Colas et al. 1996 *Nature* 380:548; and Gyuris et al. *Cell* 1993 75:791). Yeast cells are transformed with bait and prey genes. If an aptamer binds to variant A domain, the

15   LexA activation domain is brought into proximity with the LexA DNA binding domain and expression of genes having an appropriately positioned LexA binding site increases.

To test this system, yeast strain EGY48 was transformed with the bait plasmid and pSH18-34 (a URA3, 2 µm plasmid containing the GAL1 promoter fused to lacZ, in which the GAL1 enhancer-like Upstream Activating Sequence (UASG) has been replaced with

20   binding sites for eight LexA dimers). The yeast strain contains two reporter genes, LexAop-LEU2 (replaces the yeast chromosomal LEU2 gene) and LexAop-lacZ, carried on a 2µm HIS3$^+$ plasmid). Transformed cells were plated on medium containing X-gal. The presence of the bait did not cause the expression of β-galactosidase. The bait by itself also did not activate the LexAop-Leu2 gene in EGY48, since the transformed cells did not grow on Leu

25   deficient plates. Next, nuclear transport of the bait was assessed using the bait plasmid and a pJK101 reporter in a repression type assay. In this assay DNA binding by transcriptionally inert LexA fusion proteins can be detected. The pJK101 reporter is a URA3, 2 µm plasmid containing most of the UASG. A high affinity binding site for lexA is located between the Gal1 transcription start and the UASG. CD11bA-LexA bait impaired the galactosidase

activity of yeast harboring the pJK101 plasmid when grown on galactose medium, indicating that this bait enters the nucleus.

An aptamer library was introduced into the Leu and lacZ reporter-containing yeast cells. Synthesis of library proteins was induced by growing the yeast in galactose medium.

5   In the absence of a suitable prey that binds the bait, the yeast cells does not grow on Leu⁻ medium and have no β-galactosidase activity. A cell expressing a suitable prey will form colonies on Leu⁻ medium and have β -galactosidase activity. Selective galactose (but not glucose) inducible expression allows the Leu and LacZ phenotypes to be unambiguously ascribed to the library protein, diminishing the number of library plasmids that must be

10   excluded by subsequent analysis. Plasmids from positive colonies (i.e., colonies able to grow on galactose, Ura⁻, Trp⁻ His⁻ Leu⁻ plates) were rescued (Hoffman et al. 1987 *Gene* 57:267). Before the respective clones were further characterized, the specificity of their interaction with the bait was tested. This was done by showing that they do not interact with unrelated or nonfunctional baits (e.g., a CD11a A-domain-lexA and 11bA-D242A-lexA respectively),

15   with the DNA-binding domain portion of the bait or nonspecifically with the promoters or other elements of the transcription machinery. The library plasmids were rescued from the galactose-dependent Leu+ lacZ+ yeast and re-introduced into the original selection strain and into other strains containing different baits. Specific interactors confer the galactose-dependent Leu+ and lacZ+ phenotype to yeast containing the original bait, but not to yeast

20   containing unrelated baits. An interaction mating assay can be used to test specificity. Briefly, a strain that contains the prey is mated with different yeast strains which express either the original bait protein or the control bait proteins. Reporters should only be active in diploids that contain the original bait (Finley et al. (1994) Proc. Nat'l Acad. Sci USA 91:12980).

25   This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein (e.g., an variant integrin polypeptide modulating agent) in an appropriate animal model to determine the efficacy, toxicity, side effects, or mechanism of action, of treatment with such an agent. Furthermore, novel agents identified by the above-

30   described screening assays can be used for treatments as described herein.

Pharmaceutical Compositions

The variant integrin polypeptides, fragments thereof, as well as anti-variant integrin polypeptide antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions. Such compositions typically include the

5 protein or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes solvents, dispersion media, coatings, antibacterial and anti-fungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions.

10 A pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, intravenous, intradermal, subcutaneous, oral, inhalation, transdermal, transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for

15 injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with

20 acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous

25 preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the

30 contaminating action of microorganisms such as bacteria and fungi. The carrier can be a

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solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and

5   by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought

10   about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are

15   prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered

20   solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash.

25   Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as

30   magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening

agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit

containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the

5   LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit high therapeutic indeces are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such

10   compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no

15   toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which

20   achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

As defined herein, a therapeutically effective amount of protein or polypeptide (i.e., an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about

25   0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The protein or polypeptide can be administered one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. The skilled artisan

30   will appreciate that certain factors may influence the dosage and timing required to

effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of

5   treatments.

For antibodies, the preferred dosage is 0.1 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg). If the antibody is to act in the brain, a dosage of 50 mg/kg to 100 mg/kg is usually appropriate. Generally, partially human antibodies and fully human antibodies have a longer half-life within the human body than other antibodies. Accordingly, lower dosages

10   and less frequent administration is often possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (e.g., into the brain). A method for lipidation of antibodies is described by Cruikshank et al. ((1997) J. Acquired Immune Deficiency Syndromes and Human Retrovirology 14:193).

The present invention encompasses agents which modulate expression or activity.

15   An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics (e.g., peptoids), amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e,. including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or

20   inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

Exemplary doses include milligram or microgram amounts of the small molecule per

25   kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. When one

30   or more of these small molecules is to be administered to an animal (e.g., a human) in order

to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend

5   upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

An antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as

10   a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine,

15   lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and

20   cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological

25   response, the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin or a protein such as a cytokine or interleukin.

Alternatively, an antibody can be conjugated to a second antibody to form an

30   antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

Animal Models of Processes Involving Integrins

A model of vascular injury that can be useful in testing potential therapeutic compositions is described by Simon et al. (J. *Clin. Invest.* 2000 105:293). Tang et al. (1997 *J Exp Med* 186:1853) describe CD11b knockout mice which can be useful in various screening assays. An animal model of burn injury may also be useful (*Plast Reconstr Surg* 1995 96:1177).

Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant or unwanted integrin expression or activity.

"Treatment" or "treating a patient", as used herein, is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, palliate, improve or affect the disease, the symptoms of disease or the predisposition toward disease. A therapeutic agent includes, but is not limited to, small molecules, peptides, antibodies, ribozymes and antisense oligonucleotides.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is

5 the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

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